Bacterial Infections in Neonates, Madagascar, 2012–2014

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Severe bacterial infections are a leading cause of death among neonates in low-income countries, which harbor several factors leading to emergence and spread of multidrug-resistant bacteria. Low-income countries should prioritize interventions to decrease neonatal infections; however, data are scarce, specifically from the community. To assess incidence, etiologies, and antimicrobial drug–resistance patterns of neonatal infections, during 2012–2014, we conducted a community-based prospective investigation of 981 newborns in rural and urban areas of Madagascar. The incidence of culture-confirmed severe neonatal infections was high: 17.7 cases/1,000 live births. Most (75%) occurred during the first week of life. The most common (81%) bacteria isolated were gram-negative. The incidence rate for multidrug-resistant neonatal infection was 7.7 cases/1,000 live births. In Madagascar, interventions to improve prevention, early diagnosis, and management of bacterial infections in neonates should be prioritized.

Most deaths of children <5 years of age (6.3 million in 2013) still occur in low-income countries; a leading cause is infectious disease (1). In these countries, deaths of neonates are particularly concerning; in 2013, there were 20 deaths/1,000 live births, 23% directly attributable to severe infections (1–3). Each year in low-income countries, 7 million possible (clinical signs with no bacteriological documentation) severe neonatal bacterial infections occur (4,5). In these countries, multiple factors lead to enhanced emergence and spread of drug-resistant bacteria (e.g., antimicrobial drug misuse, poor quality or counterfeit drugs,

and substandard hygiene and living conditions) (6,7). This phenomenon involves gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and gram-negative (*Haemophilus influenzae*, *Enterobacteriaceae*) bacteria (8). These pathogens, especially those acquired in hospitals, are becoming increasingly resistant to multiple drugs; for most populations in these settings, the antimicrobial drugs required to treat these infections are not affordable (9).

Because few data on the burden of invasive bacterial infections and resistance patterns in low-income countries are available, we do not have an accurate picture of their true burden among the youngest children. Indeed, most studies of antimicrobial drug resistance in neonates were conducted >10 years ago. Data about antimicrobial drug resistance were sparse and often relied on few isolates; no clear conclusions have been made with regard to *Enterobacteriaceae* resistance to third-generation cephalosporins (6%–97% of infections) or methicillin resistance among *S. aureus* (0–67%) (10,11). Moreover, data regarding infections occurring in the community, which may differ from those in hospitalized persons, are especially lacking. To our knowledge, incidence rates for severe resistant infections in neonates have not been estimated (10,11).

In low-income countries, investment and mobilization to control neonatal infections and antimicrobial drug resistance remain extremely low. As long as the real burden of these events remains unknown, the scope for public health decision-making will be limited (10,12). Therefore, to assess incidence, etiologies, and antimicrobial drug–resistance patterns of neonatal infections, we conducted a prospective study of a cohort of 981 newborns enrolled at birth in rural and urban communities in Madagascar, one of the poorest countries in the world, where the mortality rate for neonates is high (13).

Methods

This study was part of the Bacterial Infections and Antimicrobial Drug Resistant Diseases among Young Children

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in Low-Income Countries (BIRDY project, http://www.birdyprogram.org). The BIRDY project investigates and responds to consequences of bacterial sepsis and antimicrobial drug resistance in children <2 years of age (protocol in online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/4/16-1977-Techapp1.pdf). The study was authorized by the Institut Pasteur in Paris and by the Ethics Committee in Madagascar. Informed consent was obtained for all participants.

Study Areas and Study Population

The study population included all neonates born in 3 districts (Avaradoha, Besarety, and Soavinadriana) of Antananarivo (the capital of Madagascar, with a catchment area population of 14,997 and 4,128 women of childbearing age) and those of the rural city of Moramanga (catchment area population of 17,159 and 3,795 women of childbearing age) (Figure 1). These areas were chosen because their populations, from poor to extremely poor, were representative of the general population.

Recruitment

Before Birth

We exhaustively identified pregnant women within the study areas during their routine third trimester antenatal visit and pre-enrolled those who met the following criteria: routine residence in the study area with no plan to move away during the follow-up period and no opposition to the research being conducted or to the collection of biological samples (online Technical Appendix). We actively monitored preincluded women to ensure enrollment of their neonates at birth. At the time of preinclusion or at delivery, a vaginal swab sample was collected from the pregnant women to detect group B *Streptococcus* (GBS).

At Birth

To ensure the exhaustiveness of live-birth recruitment, all newborns were eligible at birth, even if their mothers had not been pre-enrolled. Neonate inclusion criteria were similar to preinclusion criteria of pregnant women: neonates born to parents living in the study area with no plan to move during the follow-up period; those whose legal guardians were informed and had no objection to the study procedures and collection of biological samples; and those for whom written consent was obtained from at least 1 legal guardian.

We collected fecal samples from the mothers perinatally to test for extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae*. We also collected the mothers' sociodemographic, medical, and obstetric characteristics; delivery information; and the neonates' anthropometric measurements and Apgar scores.
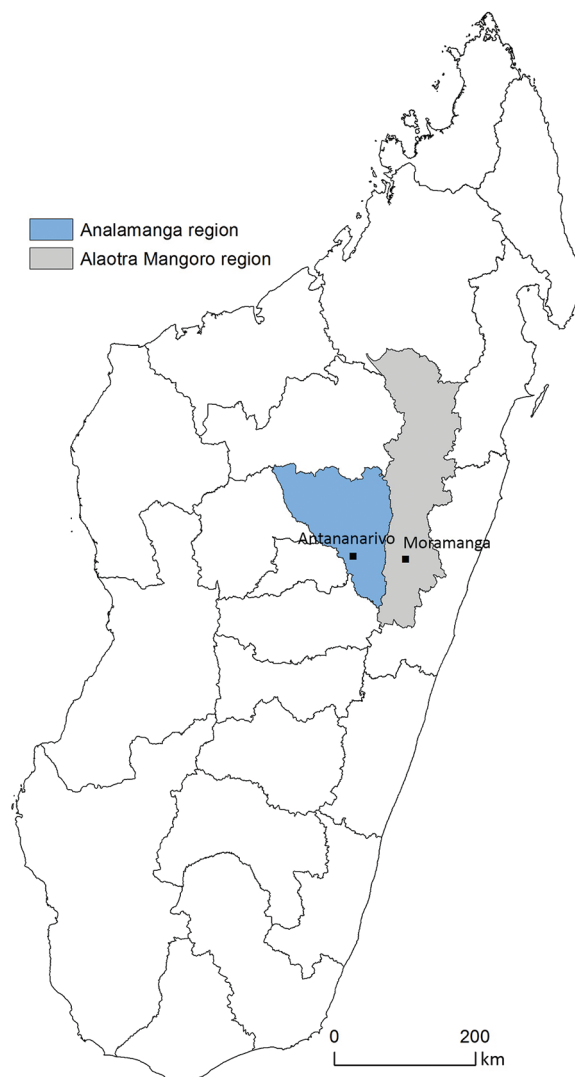

Figure 1. Locations of Antananarivo and Moramanga in Madagascar.

The neonates were examined at birth, and risk factors for infection (online Technical Appendix) were assessed. The presence of risk factors for infection led immediately to collection of a placental biopsy sample and collection of gastric fluid (before the first feeding), deep auditory canal samples, and anal swab samples from the neonate to document perinatal bacterial colonization. We then referred neonates with suspected infection to a participating hospital for evaluation. When indicated, antimicrobial drugs were empirically administered according to the World Health Organization (WHO) criteria. For these neonates, we obtained blood samples and lumbar puncture samples (if indicated) beforehand (14).

Follow-Up Evaluations

We actively and prospectively followed up on all neonates during their first month of life. To detect early signs of

infection, we arranged for home visits to be conducted twice during the first week of life, beginning within 3 days after delivery. Routine checkups were then conducted weekly during the first month. We conducted active monitoring to minimize the number of missed or uncharacterized suspected infections and to obtain anthropometric measurements. Throughout follow-up, we asked mothers to contact an investigator whenever the child had a fever or showed signs suggestive of infection (online Technical Appendix). If that occurred, the child was evaluated by a physician. When indicated, we collected samples including blood cultures according to the protocol and recorded clinical presentation, final diagnosis, and collected samples.

We adapted clinical criteria for infection and flow charts for bacterial sampling from WHO recommendations (online Technical Appendix). Decisions regarding antimicrobial drug treatments were left to the attending physicians to decide according to local protocols.

Bacteriology Analyses

All samples were transported within hours to Institut Pasteur in Madagascar for analysis. Specimen sampling, bacterial isolation, and species identification were performed according to the procedures recommended by the French Society for Microbiology (*15*). Antimicrobial susceptibilities were determined by use of the disk-diffusion method, according to the recommendations of the French Society for Microbiology (online Technical Appendix) (*15*). Suspected ESBL-producing *Enterobacteriaciae* were confirmed by use of the double-disk synergy test. *Escherichia coli* ATCC 25922 was used for quality control strains.

Classification Procedures

All cases for whom clinical or biological criteria for bacterial infection occurred during the neonatal period (including biological markers of infection based on C-reactive protein or complete blood count when available) were reviewed by an epidemiologist, a neonatologist, and a microbiologist to classify them and exclude nonsevere cases and contaminants. We defined severe bacterial infection as 1) presence of clinical signs of sepsis according to the WHO guidelines (online Technical Appendix) and 2) a positive culture from blood or cerebrospinal fluid or urine (bacterial and leukocyte counts $\geq 10^5$ and 10^4, respectively) or umbilical purulent discharge in case of omphalitis-associated sepsis. We defined 3 periods: very early (0–3 days), early (0–6 days), and late (7–30 days). We considered multidrug-resistant infections to be those caused by pathogens resistant to ≥ 1 agent in ≥ 3 antibacterial categories (*16*).

Statistical Analyses

For our analyses, we used Stata version 12 (StataCorp, LLC, College Station, TX, USA). We used descriptive statistics (e.g., proportions, means, and SDs) to summarize characteristics of mothers and neonates. We compared differences in proportions and means by using the χ^2 and Student *t* tests, respectively. p<0.05 was considered significant. We calculated the person-time (no. days followed until event [infection]) and then estimated the incidence of culture-confirmed severe neonatal infections per 1,000 live births. We calculated 95% CIs for all rates.

Results

Characteristics of Mothers and Neonates

From September 2012 through October 2014, we approached 1,030 pregnant women, of whom 54 refused to be included and 976 were enrolled (Table 1; Figure 2); of those included, 393 (40.3%) were from the urban site and 583 (59.7%) from the rural site. On average, the women were 26.1 years of age (range 14–48 years of age) and 33.7% were primigravidae. A total of 351 (37%) women gave birth at home. At delivery, 981 live neonates were included; mean ± SD birth weight was 2,952.6 ± 504.4 g; of these neonates, 161 (16%) were premature (<37 weeks' gestation).

Incidence of Neonatal Infections

A total of 16 neonates were classified as having culture-confirmed severe infection (online Technical Appendix). Of these, 12 (75%) infections occurred during the first week of life. The incidence rates were 17.7 (95% CI 10.8–28.9) culture-confirmed cases of severe neonatal infection and 13.3 (95% CI 7.5–23.4) culture-confirmed cases of early-onset severe neonatal infections per 1,000 live births. The incidence rates for culture-confirmed severe neonatal infections were 14.8 (95% CI 7.4–29.5)/1,000 live births in rural sites and 22.2 (95% CI 11.1–44.4)/1,000 live births in urban sites. The incidence rates for culture-confirmed severe neonatal infections were 15.6 (95% CI 7.0–34.6)/1,000 live births at home and 19.4 (95% CI 10.4–36.0)/1,000 live births at healthcare facilities. Final clinical diagnoses were sepsis for 13 and meningitis for 3 neonates.

Samples and Pathogens

We cultured 144 blood (including 65 [45.1%] at birth), 79 urine, and 7 cerebrospinal fluid samples from neonates with clinical signs of infection (Table 2). Among blood samples, results of 9 (6.3%) were positive and 8 (5.5%) others were considered to be contaminated. Among urine samples, results were positive for 39 (49.4%), of which 3 were associated with severe neonatal infection. One (14.3%) cerebrospinal fluid sample was culture-positive for *Pasteurella* spp., and 2 (28.6%) others grew gram-negative bacteria that could not be further identified. Gram-negative rods were detected in 13 (81.2%) samples from the 16 neonates

Table 1. Characteristics of mothers and neonates enrolled in study of bacterial infections in neonates, Antananarivo and Moramanga, Madagascar, 2012–2014

Characteristic	Urban site, no. (%)	Rural site, no. (%)	p value
Pregnant women*†			
Parity			
Primigravida	144 (37)	185 (32)	>0.99
Multigravida	249 (63)	398 (68)	
Education			
None or primary	119 (30)	145 (25)	<0.001
Partial secondary	171 (44)	334 (57)	
Completed secondary or university	103 (26)	104 (18)	
No. antenatal visits at enrollment			
0–1	45 (11)	47 (8)	0.01
2–4	239 (61)	408 (70)	
>4	109 (28)	128 (22)	
Neonates‡			
M	215 (55)	277 (47)	0.03
F	179 (45)	310 (53)	
Premature, <37 wk of gestation	77 (19)	84 (14)	0.6
Risk factors for infection at delivery			
Fetid amniotic fluid	39 (9.9)	42 (7.1)	0.13
Prolonged membrane rupture	13 (3.3)	15 (2.6)	
Maternal fever at delivery	5 (1.2)	9 (1.5)	
Difficult birth	25 (6.3)	56 (9.5)	

*Mean (± SD, minimum–maximum) age of urban mothers 25.8 (6.7, 14.3–43.5) years and of rural mothers 26.2 (6.5, 14.3–48.1) years; p = 0.4.
†Total = 976 pregnant women (393 urban and 583 rural), including 17 who had twin pregnancies and 12 who had stillbirths.
‡Total = 981 (394 urban and 587 rural). Mean (± SD) weights of 954 neonates at delivery were 2,921 (±515.9) g for urban and 2,973 (± 495.7) g for rural sites; p = 0.12.

with culture-confirmed severe infections; the most prevalent pathogen was *Klebsiella* spp.

Antibacterial Resistance

Among the 11 samples with gram-negative rods that could be tested for antimicrobial drug susceptibility, more than half showed resistance to cefotaxime (6/10) and more than one third were resistant to gentamicin (4/10) and ciprofloxacin (4/11) (online Technical Appendix Table 2). Among the 14 isolates for which antimicrobial drug resistance data were available, 5 isolates were resistant to ciprofloxacin and 9 were resistant to co-trimoxazole. Of the 6 *Klebsiella* spp. isolates, 4 were ESBL producers. The isolated *Staphylococcus epidermidis* strain was resistant to methicillin.

A total of 11 isolates were resistant to ≥1 antimicrobial drug of the combination recommended by WHO for cases of neonatal sepsis (ampicillin and gentamicin); 4 were resistant to both drugs. The incidence rates for severe neonatal infection resistant to 1 drug recommended by WHO was 7.7 (95% CI 3.7–16.2) cases/1,000 live births and to both drugs was 4.4 (95% CI 1.6–11.7) cases/1,000 live births. Seven isolates were multidrug resistant, and the incidence rate for multidrug-resistant severe neonatal infection was 7.7 (95% CI 3.7–16.2) cases/1,000 live births.

Clinical Outcomes

In total, 19 neonates, including 2 sets of twins and 1 other twin, died during the follow-up period. Four died at home with no etiology documented, 3 deaths were the direct consequence of severe prematurity, 1 was caused by birth injury, and 1 was caused by neonatal tetanus. The 10 remaining infants who died showed clinical signs of severe infection; no blood cultures could be performed before death. Six neonates were premature. All deliveries took place in healthcare facilities, except for 1, which occurred at home. The mother of a pair of twins was positive for vaginal carriage of GBS. A total of 4 neonates received a combination of gentamicin and a third-generation cephalosporin, and 5 received penicillin in addition to the 2 other drugs. All neonatal deaths except 1 occurred in the first week of life. None of the 16 neonates with a culture-confirmed severe infection died.

Discussion

Incidence of culture-confirmed severe neonatal infections in a community-based cohort of neonates in Madagascar was high (17.7 cases/1,000 live births). These infections are usually difficult to document, especially where women frequently deliver their babies at home, because neonates may show few symptoms before the infections progress rapidly. By using active community recruitment and follow-up, we were able to identify severe neonatal bacterial infections, including those with very early onset. Also, performing blood cultures before initiating antimicrobial drug therapy increased the likelihood of identifying a pathogen.

In low-income countries, incidence estimates for severe neonatal infections are few and the available data are heterogeneous (*10*). On the basis of community recruitment, Darmstadt et al. estimated an incidence rate of confirmed severe neonatal infection of 2.9 (95% CI 1.9–4.2)/1,000 live births almost 10 years ago in Bangladesh; this rate is much lower than the one we found (*17*).

However, the findings of Darmstadt et al. may be underestimated because of delayed care seeking and a shorter active surveillance period.

Our incidence estimate is lower than the 44.8 early-onset infections/1,000 live births found by Turner et al. on the Thailand–Myanmar border; their estimate was based on a clinical definition of infections and was thus possibly overestimated (*18*). Also, our incidence risk (1.6%, 16/981) is lower than the pooled incidence risk for possible severe bacterial infection (7.6%) estimated by Seale et al. in a metaanalysis of 22 studies (*5*). However, the designs of the studies contributing to the Seale analysis varied widely. Our community-based study with pre-enrollment of pregnant women before delivery is likely to reflect a higher ascertainment.

The bacterial isolation rate in blood culture is low (≈10%) for infected neonates in high-income and low-income countries (*19*). Blood cultures require that trained staff collect these samples before any antimicrobial drug use is initiated. These practices are not routine in low-income countries, and some highly suspected infections could not be bacteriologically confirmed, even in the setting of our research protocol, which included continuous training. However elevated, the incidence rate of confirmed severe infections may therefore be underestimated in our study.

As a comparison, in 2008, the United States reported 0.77 early-onset infections/1,000 live births (*20*). Although most studies in high-resource settings focus on the early neonatal period and are not population based, our results clearly suggest a much higher burden of neonatal infections in Madagascar than in high-income countries.

Most (75%) neonatal infections occurred during the first week after birth, most during the first 3 days. This finding confirms that community-based active surveillance in the very early period of life is crucial for capturing infections in neonates (*4*). This result also points out the value of reinforcing interventions and research programs targeting the perinatal period.

Gram-negative bacteria were predominant; the most prevalent pathogen isolated was *Klebsiella* spp. In a review of studies reporting the etiology of serious bacterial infections in community settings, Zaidi et al. found that *Klebsiella* spp., *E. coli*, and *S. aureus* were the most prevalent bacteria isolated during the first week of life (*21–25*). In our study, *S. aureus* was not predominant. It is possible that healthcare workers caring for mothers and neonates in our study were more prone to use clean birth kits distributed by the BIRDY program and to follow good hygiene practices, potentially minimizing horizontal transmission of *S. aureus* to newborns.

The overall burden of GBS infection in the developing world is not clear; incidence ranges from 0.3 to 0.6
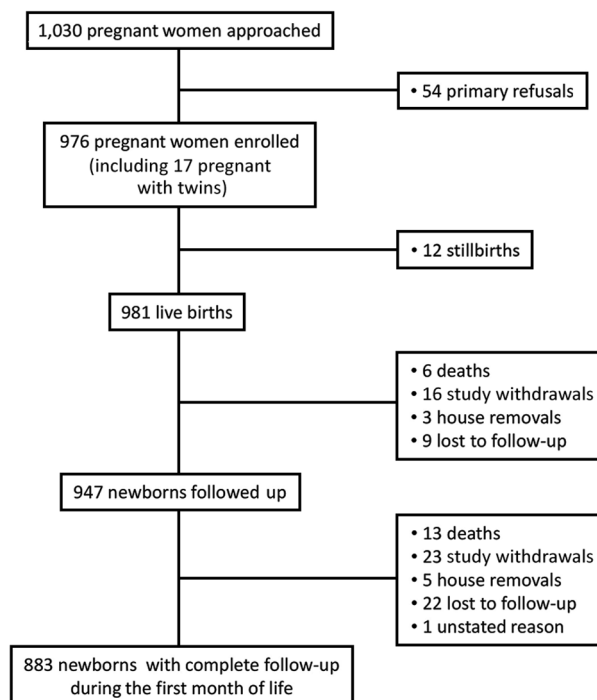

Figure 2. Flowchart for study of bacterial infections in neonates, Antananarivo and Moramanga, Madagascar, 2012–2014.

infections/1,000 live births (*26*). Our study identified no GBS infections. One possible explanation for this low incidence is that several early-onset GBS infections may have not been identified because of rapid death (*27*). However, this hypothesis is unlikely because we performed close and active surveillance directly after birth and no deaths occurred during the very short period between delivery and neonate enrollment. However, we cannot exclude the possibility that GBS might have been responsible for some cases of infection that could not be bacteriologically confirmed for neonates with clinical signs of sepsis, including some who died. In the context of GBS vaccine development, if confirmed, this low incidence may bring into question the potential cost-effectiveness of maternal vaccination in low-income countries.

We found that the proportion of multidrug-resistant infections was significant (50%, 7/14); 28.6% (4/14) of *Enterobacteriaceae* were ESBL producers, and 1 of the 2 *Staphylococcus* spp. isolates was resistant to methicillin. One striking result of our study, however, is the relatively low incidence of antimicrobial drug–resistant infections (≈7.7 infections/1,000 live births). We found no carbapenemase-producing *Enterobacteriaceae*. In most published studies, assessment of antimicrobial drug resistance at the community level is based on the proportion of resistant infections at hospital admission, which may lead to biased conclusions because of variability in care access

Table 2. Pathogens isolated and characteristics of neonates with culture-confirmed severe infections, Antananarivo and Moramanga, Madagascar, 2012–2014*

Pathogen	No. isolates	Neonate age at infection, d				Sample type, no.	Place of birth		Premature		Site	
		0–3	4–6	7–13	14–30		Home	HCF	No	Yes	Urban	Rural
Gram-positive												
Staphylococcus aureus	1				1	Blood, 1	1			1	1	
S. epidermidis	1				1	Blood, 1	1			1	1	
Streptococcus pneumoniae	1				1	Blood, 1	1		1		1	
Gram-negative												
Klebsisella pneumoniae	4	1	3			Blood, 2; urine, 2	1	3	4		1	3
K. oxytoca	2	1	1			Blood, 1; urine, 1		2	2		2	
Escherichia coli	2	2				Blood, 2	1	1	1	1		2
Enterobacter cloacae	1	1				Blood, 1	1		1	1		1
Acinetobacter baumanii	1	1				Umbilical, 1		1	1			1
Pasteurella spp.	1				1	Spinal tap, 1		1	1			1
Gram-negative staining†	2	2				Spinal tap, 2		2	2	2		2

*HCF, healthcare facility.
†Pathogens identified by staining as gram-negative but could not be cultured and, therefore, were not further identified.

and case severity. Our results enabled a more complete picture of this issue and suggest that multidrug-resistant infections in the community may be less problematic than previously estimated.

Nevertheless, more than three quarters (11/14) of the pathogens we isolated were resistant to at least 1 antimicrobial drug recommended by WHO, including 4 isolates resistant to both recommended drugs (*14*). These findings are consistent with those of several studies conducted in hospital or community settings, which also highlight reduced susceptibility to at least 1 antimicrobial drug recommended for empirical treatment (resistance ranging from 43% to 97%) (*19,22,28*). In contrast, we observed that the most frequent attitude of physicians in Madagascar was to prescribe 3 antimicrobial drugs, including ampicillin, a third-generation cephalosporin, and gentamicin, when invasive bacterial neonatal infection was suspected. The use of large and unnecessarily broad-spectrum therapy may contribute to increased rates of antimicrobial drug resistance. The development of rapid diagnostic tests to identify pathogens and their antimicrobial drug susceptibility may therefore prevent unnecessary use of broad-spectrum antimicrobial drugs.

Our study has some limitations. We cannot exclude the possibility that 4 pathogens, including one ESBL-producing *Enterobacteriaceae*, which we documented in the community, might have been acquired in the hospital because the infections occurred after 48 hours in neonates born in a healthcare facility. However, no hospitalization during pregnancy was recorded for the mothers of any of these 4 neonates. Also, because the neonates were enrolled in a research study, their standard of care might have been higher, including better hand hygiene, than that for most of the population. This Hawthorne effect might have induced bias in our results, such as an underestimation of *Staphylococcus*-associated infections (*29*). We also probably changed the evolution of these severe bacterial infections by improving early diagnosis and providing better care. These actions might have helped avoid deaths of neonates, which would otherwise have occurred, and contributed to our underestimation of case-fatality ratio.

In conclusion, incidence of bacterial infections among neonates in a community-based cohort in Madagascar was high, although incidence of multidrug-resistant bacterial infections was relatively low. Most of these infections occurred during the first week of life. Our findings suggest that public health measures to decrease deaths from severe bacterial infection among neonates should focus on improving prevention, early diagnosis, and management of infections and prioritizing intervention strategies according to successes with vaccines, clean deliveries, and care of neonates. Current knowledge gaps, including those associated with local burden, bacterial etiology, and antimicrobial drug resistance profiles of severe bacterial infections in low-income countries, prevent us from having a clear picture of the situation. Recently, several international bodies called for global action to combat antimicrobial drug resistance, deemed a "global health security threat" (*11,30*). Although antimicrobial drug resistance is a real threat, more community data are clearly needed in countries with limited resources so they can select and prioritize effective preventive and treatment strategies to tackle bacterial infections in neonates.

About the Author

Dr. Huynh is a medical epidemiologist at Institut Pasteur. Her interests lie in applied public health research, and her main research topics focus on maternal and child health in low-income countries, specifically, the causes and consequences of malaria during pregnancy in sub-Saharan Africa, infections in neonates, and antimicrobial resistance in low-income countries.
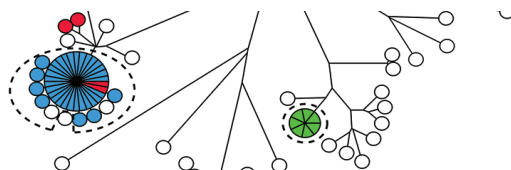
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Bacterial Infections in Neonates, Madagascar, 2012–2014

Technical Appendix

Section 1: Methodology of the BIRDY program

1. Development within a site

In each site, the program has two distinct phases. The first is **a pilot phase lasting approximately 1 year.** This phase aims at solving possible issues **regarding** data collection, organization, and logistics, before planning the full investigation, to begin during the second year.

Therefore, the specific objective of the pilot phase is to assess the feasibility of the study, at several sites, in real-life situations and to identify potential implementation problems and to find the solution to these problems.

During the pilot phase, we plan to recruit circa 1000 live born neonates at each site, combining both rural and urban setting.

After the pilot phase, the program continues into the complete program using the same geographic basis, with the aim of recruiting ≈1000 live births per year.

2. Study population and recruitment sources

The study population consists of neonates followed from birth until the age of 2 years. Two periods have been identified for recruitment: at birth, or before birth in the so–called "pre-inclusion" phase.

3. General description of the cohort

The general organization of the investigation is illustrated in Technical Appendix Figures 1 and 2.

3.1 Recruitment (see Technical Appendix Figure 1)

Pregnant women

At each site, we first organize the exhaustive identification of pregnant women within a geographically based defined population. Each pregnant woman is asked to participate in the study.

Pregnant women are systematically provided with information about the project during prenatal consultations.

Pre-inclusion occurs during third trimester consultation for all women meeting the pre-inclusion criteria (see Section 2) and giving informed written consent.

At the time of pre-inclusion, the "maternal" section of the electronic Clinical Report Form (e–CRFm) concerning the socio-demographic, medical, and obstetric characteristics of the mother is completed.

The neonates born from the pre-included mothers are included in the study at the time of delivery.

Given the main objective of the study, which relates to the measurement of the incidence of bacterial infections, and the potentially large number of women who do not have antenatal consultations, we optimize the exhaustiveness of live birth recruitment by also including neonates at the time of birth, without a prior pre-inclusion phase.

Births are much easier to identify for deliveries in healthcare structures participating in this project. However, because of a high proportion of home births, particularly in rural environments, we ensure that women giving birth outside healthcare structures are identified as rapidly as possible, through regular monitoring of mothers around the predicted date of delivery, by using text messages and mobile phones and by involving community workers, particularly in rural environments, and working with the matrons.

Investigators seek information daily from healthcare structures and matrons participating in the project concerning any births occurring over the last 24 hours.

To help ensure that the recruitment of the children is exhaustive, we also compare our data with the official birth registry where possible.

At birth

Neonates are included in the study if they meet the inclusion criteria (see Section 2).

A rectal and a vaginal swab sample is taken in mothers giving birth in a healthcare structure.

The e–CRFm is completed at the time of the delivery if the mother has not already been pre-included, and the neonate is thus recruited at the time of delivery.

At the time of birth, an initial "child" e–CRFc concerning the characteristics of the delivery is completed. Anthropometric measurements (weight, length, head circumference and brachial circumference) are recorded. An APGAR score is obtained by the care staff handling the delivery, one, five, and 10 minutes after delivery. The neonate is examined by a health care worker who checks the presence of risk factors for infection (Section 3).

If the mother gives birth with the assistance of a matron (not in a health care structure) the investigator is notified as soon as possible, for the collection of medical information from the matron concerning the birth and the neonate. The investigator checks the presence of risk factors for infection. Babies at risk for infection are referred to a participating hospital for pediatric evaluation. If the neonate dies before the arrival of the investigator, the e–CRFc is completed and a verbal autopsy is carried out by the investigator with the matron and, if possible, the mother or a relative.

The presence of a risk factor for infection at birth systematically leads to collection of the following samples within few hours after birth: gastric fluid (before the first feed), deep auditory canal swabs, anal swabs and a placental biopsy[1].

The decision as to whether to administer an empirical antibiotic treatment at birth is made in accordance with WHO criteria. If the decision is made to begin empirical antibiotic treatment, blood cultures and blood sampling for CRP determination is performed beforehand in addition to the collection of the systematic samples. A chest X- ray and lumbar puncture may also be requested, at the discretion of the clinician, according to the clinical context.

3.2 Follow–up (see Technical Appendix Figure 2)

General organization

After their births the children are followed for the first 2 years of their lives. Follow-up is both passive and active.

Throughout the follow–up period, passive follow–up consists in asking the mother to contact an investigator whenever the child has fever or meets criteria suggestive of infection (Section 3). An information leaflet describing these criteria is distributed and explained to the mothers beforehand. The mothers are also provided with a thermometer and asked to check the child's axillary temperature. Body temperature is monitored daily during the first month of life, and then weekly for the rest of the follow–up period. The temperatures are recorded on a paper document (which is adapted appropriately if the mother is unable to read).

Close active follow–up is also carried out to minimize the number of missed or uncharacterized infections. During the first 7 days of life, an update concerning the child is requested daily by sending a text message to a mobile telephone. In addition, two home visits are planned, the first one within 3 days of delivery. During this initial visit, the investigator determines the gestational age of the newborn using "Ballard" score. (The Ballard Maturational Assessment, Ballard Score, is a commonly used technique of gestational age assessment. This involves a clinical examination of the neonate evaluating physical and neurologic maturity. It assigns a score to various criteria, the sum of all of which is then extrapolated to the gestational age of the baby. These criteria are divided into Physical and Neurologic criteria. This scoring allows for the estimation of gestational age in the range of 26 weeks–40 weeks.)

Thereafter, routine check–ups take place weekly during the first month of life, then fortnightly until 3 months of age and monthly between 3 months and twelve months and then every 2 months between 1 and 2 years of age. These check-ups are carried out by investigators and makes it possible to note the occurrence of infectious episodes that have not already been identified, to detect possible infections at the time of the check-up, to remind the mother of the importance of the continuing participation of her child in the cohort, and to provide useful information for the follow–up of the child. The child is also weighed and measured (height, brachial circumference, and head circumference).

All the investigators participating in this project are trained in techniques for weighing the children, taking anthropometric measurements, determining "Ballard" scores and in the clinical evaluation of infection criteria. Furthermore, regular checks are made to ensure the correct execution of the various measurement techniques.

3.3 Presence of infection criteria during follow-up (see Technical Appendix Figure 3)

A criterion for infection may be detected by the mother, who may then consult at the hospital or the Primary Care Center (PCC), or call the investigator. The temperature chart completed by the mother is shown to the investigator during his or her visit, or to the staff of the health center or hospital in cases of direct consultation.

A criterion for infection may be observed by the investigator during routine check–up visits or during a visit at the request of the mother.

When fever is confirmed (axillary temperature ≥37.5C) or in the presence of other clinical criteria for infection, the child is to be examined by a medical doctor (at the reference/district hospital or at the PCC). If the investigator has followed initial basic paramedical or medical training, he or she may determine whether the state of health of the child requires immediate hospitalization or whether the treatment at the PCC is possible. If the investigator has no basic paramedical or medical training, he or she sends the child directly to hospital if a criterion of infection is detected.

The signs and symptoms of the child, the final diagnosis and the samples taken are recorded in the e–CRFc.

The medical evaluation is carried out by the attending medical doctor, who completes a paper questionnaire. At the time of data entry, the investigator checks that the collected information is complete, and adds any missing information, with the assistance of the doctor, when necessary:

- Systematic samples in cases in which clinical criteria for suspected infection are identified: urine samples for cytobacteriological examination; blood for blood cultures; lumbar puncture in febrile children under the age of 3 months; and thick blood smears if the child has a

fever in a malaria-endemic area. Blood formula and C–reactive protein (CRP) determinations are also carried out.

- Samples based on the presence of particular warning signs of infection: stool samples for coproculture in cases of diarrhea (at least three liquid stools per day); lumbar puncture in the presence of neurologic signs or convulsions; swabbing in cases of discharge from the eyes or ears or in cases of signs of omphalitis (swabbing of the pus).

When possible, a chest x-ray is carried out in the presence of respiratory signs, at the discretion of the attending clinician. Samples for bacteriological analysis are transported as rapidly as possible in a cold box encased in secure packaging to the microbiological laboratory of the Institut Pasteur of each site.

Section 2: Criteria for inclusion and exclusion

Preinclusion of the women during pregnancy

Preinclusion criteria:

- Routine residence in the study zone of a participating country

- No plans to move away from the study zone during the period of follow-up for the neonate

- Information provided about the way in which the study will be carried out and about the collection of biologic samples from the neonate

- No opposition from the pregnant woman to the research being carried out or to the collection of biologic samples

- Signed informed consent form.

Exclusion criteria:

- Residence outside the study zone of a participating country

- Plans to move away from the study zone during the follow-up period of the neonate

- No information provided about the way in which the study is carried out or about the collection of biologic samples from the neonate

- Opposition from the woman to the research being carried out or to biologic samples being collected from the neonate.

Inclusion of the neonate at delivery

Inclusion criteria:

- Neonate born to parents living in the study zone of a participating country

- Parents of the neonate not intending to move away from the study zone during the follow-up period

- Legal guardians of the neonate informed about the way in which the study is to be carried out and about the collection of biologic samples

- Legal guardians of the neonate having no objection to the collection of biologic samples

- Authorization from at least one of the legal guardians of the child, in the form of a signed informed consent form.

Exclusion criteria:

- Stillborn neonate

- Parents of the neonate living outside the study zone of a participating country

- Neonate born to parents planning to move away from the study zone of a participating country during the follow-up period

- At least one of the legal guardians of the neonate not informed about the study or about the collection of biologic samples

- At least one of the legal guardians of the neonate opposed to the collection of biologic samples.

- Neonate already participating in another biomedical study.

NB: being below the age of majority is not considered a criterion for the non-preinclusion of a mother or her infant. However, in such cases, informed consent must be obtained from the mother herself and from one of her parents or legal guardians.

Section 3A: Screening criteria for suspected infection

At birth

Risk factors for infection at birth leading to perinatal sampling and medical evaluation (risk factors are checked by the matron or the midwife in case of home birth and by the midwife or the attending doctor in case of hospital birth):

- Unplanned preterm delivery (<37 weeks gestation)

- Prolonged membrane rupture (≥12 h)

- Maternal fever (axillary temperature >37.9 C) at the time of delivery

- Low birthweight (<2500 g)

- Difficult birth (birth asphyxia)

- Foul-smelling amniotic fluid

- Infection in a twin

- Leukorrhoea or untreated urinary infection during pregnancy

- Home birth

During the neonatal period (0–28 days):

Criteria for suspected infection leading to medical evaluation and bacterial sampling (criteria used by healthcare agents):

- Feeding difficulties

- Restlessness, irritability

- Lethargy, movement only when stimulated, hypotonia, coma

- Bulging fontanelle

- Convulsions

- Abdominal distension

- Paleness or gray skin

- Redness around umbilicus or purulent discharge from the umbilicus

- Prolonged capillary refill (>3s)

- Respiratory rate >60/min

- Apnoea (>15s) or bradypnoea (respiratory rate <20/min)

- Difficult breathing (grunting or severe chest indrawing)

- Cyanosis

- Hypothermia (<35.5C)

- Fever (axillary temperature >37.5C)

- Purulent discharge from the eyes

- Marked jaundice

- Many or severe skin pustules

Section 3 B: Criteria of infection (adapted from the WHO criteria)

During the neonatal period (0–28 days):

- Feeding difficulties

- Restlessness, irritability

- Lethargy, movement only when stimulated, hypotonia, coma

- Bulging fontanelle

- Convulsions

- Abdominal distension

- Paleness or gray skin

- Redness around umbilicus or purulent discharge from the umbilicus

- Prolonged capillary refill (>3s)

- Respiratory rate >60/min

- Apnoea (>15s) or bradypnoea (respiratory rate <20/min)

- Difficult breathing (grunting or severe chest indrawing)

- Cyanosis

- Hypothermia (<35.5C)

- Fever (axillary temperature >37.5C)

- Purulent discharge from the eyes

- Hepato- or splenomegaly

- Abnormal pulmonary auscultation

- Marked jaundice

- Many or severe skin pustules

Only severe bacterial infections were considered to evaluate incidence rates. Severe bacterial infections were defined as meningitis, pneumonia, or sepsis, irrespective of its etiology, based on the presence of clinical signs predicting severe bacterial illness from the WHO Young Infants Clinical Signs Study (in bold characters) (Young Infants Clinical Signs Study Group. Clinical signs that predict severe illness in children under age 2 months: a multicenter study. Lancet 2008; 371: 135–42).

After the age of 28 days:

- Changes in behavior

- Feeding difficulties

- Restlessness, irritability

- Lethargy, movement only when stimulated, hypotonia, coma

- Bulging fontanelle

- Convulsions

- Abdominal distension

- Paleness or gray skin

- Prolonged capillary refill (>3s)

- Respiratory rate >60/min

- Apnoea (>15s) or bradypnoea (respiratory rate <20/min)

- Difficult breathing (grunting or severe chest indrawing)

- Cyanosis

- Abnormal pulmonary auscultation

- Hypothermia (<35.5C)

- Fever (axillary temperature >37.5C)

- Marked jaundice

- Purulent ear discharge

- Purulent eye discharge

- Redness around umbilicus or purulent discharge from the umbilicus

- Painful, swollen joints

- Diarrhea, vomiting

- Hepato- or splenomegaly

- Poor weight gain (unless isolated)

- Many or severe skin pustules, skin rash

Criteria for empirical antibiotic treatment at birth[7]

- Presence of one or several clinical signs consistent with infection (see above (*3*))

- If the child is asymptomatic during the neonatal period: signs suggestive of chorioamnionitis (at least two of the following signs: prolonged membrane rupture (≥24 h); maternal fever (>37.9C) at the time of delivery; foul-smelling amniotic fluid) or infection in a twin

Section 4: Microbiological procedures

Blood cultures

Incubation was done at $35 \pm 2°C$ up to 5 days if an automated device was used or 7 days if a manual procedure was applied. In case of manual procedure and use of a two-phase impregnation bottle, a daily inspection supported by the spread of liquid medium on a solid medium were carried out.

If positive, a direct microscopic examination of the broth and Gram staining were performed.

Cultures

Inoculation of broth and /or colonies on fresh blood agar and chocolate media under a 10% CO_2 atmosphere at $35 \pm 2°C$ for 24 hours was done.

If multi-microbial growth occurred, then selective media were used.

Identification of isolated colonies was made with API galleries. If *Staphylococcus aureus* was suspected, identification was performed by coagulase and agglutination tests (Pastorex®).

Susceptibility testing was done according to CASFM guidelines.

Screening of microorganisms

Screened bacteria were *Haemophilus influenzae*, *Streptococcus pneumoniae* and *agalactiae*, *Enterococcus* spp., *Staphylococcus aureus* and coagulase negative, *Neisseria meningitidis*, Enterobacteriacea and, non-enterobacteria gram negative bacilli.

Urine cyto-bacteriology tests

Macroscopic examination is followed by microscopic examination.

A leukocyte count (/mL) with a Malassez cell (or Nageotte) on homogenized urine (threshold 10^4/mL) was performed followed by a Gram staining on unspun urine.

Cultures

Inoculation on selective media was calibrated with a loop of 10μl. Culture media were incubated at $35 \pm 2°C$ for 24 hours (optionally 48 h).

Identification of isolated colonies was made with API galleries. If *Staphylococcus aureus* was suspected, identification was performed by coagulase and agglutination tests (Pastorex®). Susceptibility testing was done according to CASFM guidelines.

Screening of microorganisms

Screened bacteria were *Escherichia coli*, *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Enterococcus* spp., *Streptococcus agalactiae*, *Staphylococcus aureus* and *saprophyticus*, *Candida* spp. Urine specimens were stored at +4°C until the release of results.

Cerebral spinal fluid cultures

Macroscopic examination classified CSF samples in clear, hemorrhagic, cloudy or citrine.

CSF microscopic examination

Erythrocyte/leukocyte counts were performed after homogenization with a counting chamber (Malassez). Quantitative cytology was done after cytocentrifugation, and Gram staining allowed tovisualize the microbial flora.

Soluble antigen detection included *Haemophilus influenzae type B*, *Streptococcus pneumoniae*, *Neisseria meningitidis* (serogroups A, C and W/Y), *Streptococcus agalactiae* and *Escherichia coli* K1.

Cultures

Two drops of CSF were spread on a chocolate agar supplemented with polyvitex and a blood agar medium and were inoculated into a Brain-Heart-Infusion (BHI) broth. These inoculated media were incubated at 36 ± 1°C with 10% CO_2 (except BHI) for 5 days.

Identification of isolated colonies was made with API galleries. If *Staphylococcus aureus* was suspected, identification was performed by coagulase and agglutination tests (Pastorex®). Susceptibility testing was done according to CASFM guidelines.

Screening of microorganisms

Screened bacteria were *Haemophilus influenzae*, *Streptococcus pneumoniae* and *agalactiae*, *Neisseria meningitidis*, *Escherichia coli* K1, *Klebsiella* spp., *Listeria monocytogenes* (aerobic incubation), *Staphylococcus* spp.

Culture-negative CSF with strong suspicion of meningitis were stored at −80°C.

Stools

A calibrated amount of stools was undertaken at baseline and reproduced throughout the project so as to use semiquantitative methods in bacteria counts.

Microscopic examination included direct examination and examination after staining with methylene blue and Gram.

Cultures

Some media were used systematically, Bromo-Cresol-Purple (BCP), Eosin-Methylene-Blue (EMB), Hektoen, Mueller-Kaufmann broth to screen for pathogenic enterobacteria, mainly *Escherichia coli*, *Salmonella* and *Shigella*.

In case of a bloody stool, a Cefsulodin-Irgasan-Novobiocin (CIN) agar was added.

Based on microscopic examination observation, additional solid media could be used, Blood agar supplemented with nalidixic acid for *Staphylococcus.aureus*, Thiosulfate-Citrate-Bile-Saccharose (TCBS) for *Vibrio* spp., and Chromagar Candida for yeast detection.

Media were incubated at $35 \pm 2°C$ (except CIN) for 24 to 48 hours.

Identification of isolated colonies was made with API galleries. If *Staphylococcus aureus* was suspected, identification was performed by coagulase and agglutination tests (Pastorex®).

Susceptibility testing was done according to CASFM guidelines.

Serotyping

Salmonella were serotyped according to the Kaufmann-White scheme, *E. coli* O157:H7 and Shigella were serotyped with the appropriate antiserums according to the identified species.

Pus

Macroscopic examination was followed by microscopic examination which includes Gram staining to detect polymorphonuclear leukocytes and to examine the composition of bacterial flora.

Cultures

Pus were spread on chocolate agar, blood agar, Chapman and BCP.

In case of anaerobic suspicion, Schaedler agar and broth were inoculated. Incubation was carried

out at $35 \pm 2°C$ with or without CO_2.

Identification of isolated colonies was made by API galleries. If *Staphylococcus aureus* was suspected, identification was performed by coagulase and agglutination tests (Pastorex®). Susceptibility testing was done according to CASFM guidelines.

Screening of microorganisms

Screened bacteria were *Staphylococcus aureus*, *Streptococcus β-hemolytic*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., Serratia spp., *Acinetobacter* spp., *Alcaligenes faecalis*, *Stenotrophomonas maltophilia*, *Neisseria gonorrhoeae*.

Perinatal samples (placental, gastric fluid, deep auditory canal swabs, rectal swab)

Microscopic examination by Gram stain.
Cultures

Samples were inoculated on Blood Agar, chocolate Agar supplemented with Polyvitex (incubated under 10% CO_2) and on enterobacteria media when influenced by Gram staining observation. Monomorphic colonies were identified. Identification of isolated colonies was made with API galleries. If *Staphylococcus aureus* is suspected, identification was performed by coagulase and agglutination tests (Pastorex®). Susceptibility testing was done according to CASFM guidelines.

Screening of microorganisms

Screened bacteria were Enterobacteriaceae, *Staphylococcus aureus*, β-hemolytic streptococci, *Enterococcus* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Listeria monocytogenes*

Technical Appendix Table 1. Clinical signs presented by 16 neonates with culture confirmed severe bacterial infection, Antananarivo and Moramanga, Madagascar, 2012–2014

Clinical signs	No. (%)
Hypothermia (<35.5°C)	3 (19)
Fever (axillary temperature >37.5°C)	7(74)
Feeding difficulties	4 (25)
Restlessness, irritability	7 (44)
Lethargy, movement only when stimulated, hypotonia, coma	6 (38)
Bulging fontanelle	1(6)
Paleness or gray skin	3 (19)
Redness around umbilicus or purulent discharge from the umbilicus	5 (31)
Prolonged capillary refill (>3s)	3 (19)
Respiratory rate >60/min	3(19)
Difficult breathing (grunting or severe chest indrawing)	3 (19)

Clinical signs	No. (%)
Cyanosis	2 (12)
Marked jaundice	4 (25)
Many or severe skin pustules	1(6)

Technical Appendix Table 2. Antimicrobial drug susceptibility of pathogens isolated from neonates with severe culture-confirmed infections, Antananarivo and Moramanga , Madagascar, 2012–2014*

Pathogen	AMP	AMC	TIC	GEN	AMK	TZP	TMP/SXT	CEF	FOX	CTX	CAZ	CIP	ERY	IPM	CHL	TET	OXA	VAN	TEC
Gram-positive																			
Staphylococcus aureus				1/0			1/0	1/0				1/0			1/0	1/0		1/0	1/0
Staphylococcus epidermidis				1/1			1/1	1/1				1/0	1/0		1/1	1/1			1/0
Streptococcus pneumoniae	1/0			1/1			1/0					1/0	1/0		1/0	1/1	1/0	1/0	1/0
Gram-negative																			
Klebsiella pneumoniae	4/4	4/3		4/3	4/0	3/0	4/4	4/3	4/0	4/3		4/2		4/0	4/2				
Klebsiella oxytoca	2/2	2/2	1/1	2/1	2/0	2/0	2/2	2/2	2/1	2/2		2/1		2/0	2/2				
Escherichia coli	2/2	2/0		2/0	2/0	1/0	1/1	2/0	2/0	2/0		2/0		2/0	2/0				
Enterobacter cloacae	1/1	1/1	1/1	1/0	1/0	1/0	1/0	1/1	1/1	1/1		1/1		1/0	1/0				
Acinetobacter baumanii		1/0†	1/0	1/0			1/1					1/0	1/0	1/0	1/0	1/1			
Pasteurella spp.				1/0‡						1/0		1/0				1/0			

*AMC, amoxicillin/clavulanate; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CEF, cephalothin; cefalotin; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; IPM, imipenem; OXA, oxacillin; TEC, teicoplanin; TET, tetracycline; TIC, ticarcillin; TMP/SXT, cotrimoxaxole; TZP, piperacillin/tazobactam; VAN, vancomycin. Data expressed as total no. isolates/no. resistant isolates.
†Ticarcillin/clavulanate.
‡Spectinomycin.

Technical Appendix Figure 1. Recruitment steps.

Technical Appendix Figure 2. Follow-up.

Recruitment of mothers during pregnancy/last prenatal consultation

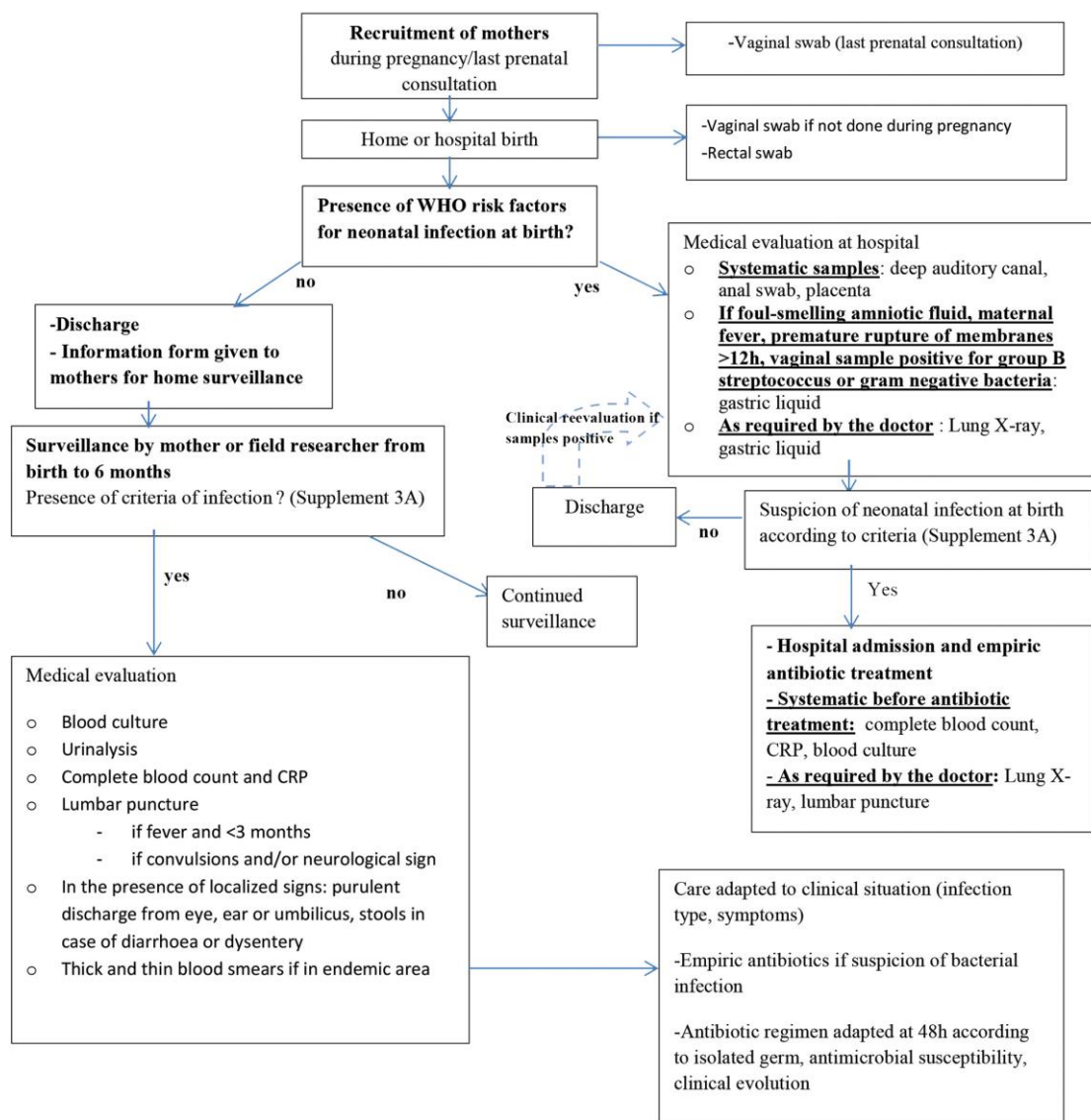→ -Vaginal swab (last prenatal consultation)

Home or hospital birth

→ -Vaginal swab if not done during pregnancy
-Rectal swab

Presence of WHO risk factors for neonatal infection at birth?

no →

-Discharge
- Information form given to mothers for home surveillance

↓

Surveillance by mother or field researcher from birth to 6 months
Presence of criteria of infection? (Supplement 3A)

yes ↓   **no** → Continued surveillance

Medical evaluation

o Blood culture
o Urinalysis
o Complete blood count and CRP
o Lumbar puncture
   - if fever and <3 months
   - if convulsions and/or neurological sign
o In the presence of localized signs: purulent discharge from eye, ear or umbilicus, stools in case of diarrhoea or dysentery
o Thick and thin blood smears if in endemic area

→

yes →

Medical evaluation at hospital
o **Systematic samples**: deep auditory canal, anal swab, placenta
o **If foul-smelling amniotic fluid, maternal fever, premature rupture of membranes >12h, vaginal sample positive for group B streptococcus or gram negative bacteria**: gastric liquid
o **As required by the doctor**: Lung X-ray, gastric liquid

Clinical reevaluation if samples positive

Discharge ← **no** ← Suspicion of neonatal infection at birth according to criteria (Supplement 3A)

Yes ↓

- **Hospital admission and empiric antibiotic treatment**
- **Systematic before antibiotic treatment:** complete blood count, CRP, blood culture
- **As required by the doctor:** Lung X-ray, lumbar puncture

Care adapted to clinical situation (infection type, symptoms)

-Empiric antibiotics if suspicion of bacterial infection

-Antibiotic regimen adapted at 48h according to isolated germ, antimicrobial susceptibility, clinical evolution

Technical Appendix Figure 3. Flow-chart for diagnosis and care of infants and newborns.